Virologisches Institut
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. sc. nat. ETH Cornel Fraefel

Arbeit unter wissenschaftlicher Betreuung von
PD Dr. med. vet. Claudia Bachofen

Rapid and simple colorimetric loop-mediated isothermal amplification (LAMP) assay for the detection of bovine alphaherpesvirus 1

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Deborah Peltzer

Tierärztin
von Basel (BS)

genehmigt auf Antrag von

PD Dr. med. vet. Claudia Bachofen, Referentin
Apl. Prof. Dr. med. vet. Martin Kaske, Korreferent

2020

CONTENTS

ZUSAMMENFASSUNG

Als Erreger der Infektiösen Bovinen Rhinotracheitis (IBR) und Infektiösen Pustulösen Vulvovaginitis/Balanoposthitis (IPV/IPB) ist das bovine Alphaherpesvirus 1 (BoHV-1) weltweit für hohe wirtschaftliche Verluste in der Rinderzucht und -mast verantwortlich.

Ziel dieser Studie war die Etablierung eines schnellen, kolorimetrischen loop-mediated isothermal amplification (LAMP) Assay für den Nachweis viraler DNA. Phenolrot wurde dazu als pH-sensitiver Indikator verwendet, der auf einem Farbumschlag von pink zu gelb im Fall einer positiven Reaktion beruht. Diverse LAMP Primer wurden verglichen und neu designte Primer erzielten dabei die schnellsten Resultate. Sie ermöglichten eine Auswertung nach 8-30 Minuten. LAMP zeigte weniger Kreuzreaktionen mit anderen Wiederkäuerherpesviren als die qPCR, aber war zehnmal weniger sensitiv. Die Detektionslimite lag aber dennoch bei nur 14 Viruskopien. Die Performance wurde anhand von 26 BoHV-1 positiven und negativen Nasentupfern von Rindern mit Atemwegserkrankung evaluiert. Bei Verwendung von Säulen-extrahierter DNA wurden alle Proben korrekt identifiziert. Mittels einfacher DNA-Präzipitationsmethode ergaben nur zwei schwach-positive Proben unklare Resultate.

Die Kombination aus DNA-Präzipitation und Behelfs-Wasserbad, erhitzt durch einen Tauchsieder, ermöglichte eine ressourcenorientierte Anwendung des Assay. Er kann daher bei der Bewältigung von IBR/IPV Ausbrüchen, wo keine teure Laborausrüstung zur Verfügung steht, äußerst wertvoll sein.

Keywords: kolorimetrischer LAMP Assay; bovines Alphaherpesvirus 1 (BoHV-1); Infektiöse Bovine Rhinotracheitis (IBR); schneller Nachweis; ressourcenorientierte Anwendung

ABSTRACT

As the causative agent of Infectious Bovine Rhinotracheitis (IBR) and Infectious Pustular Vulvovaginitis/Balanoposthitis (IPV/IPB), bovine alphaherpesvirus 1 (BoHV-1) is responsible for high economic losses in the cattle industry worldwide.

This study aimed to establish a fast, colorimetric loop-mediated isothermal amplification (LAMP) assay for the detection of viral DNA. Phenol red is used as pH-sensitive readout, relying on a distinct color change from pink to yellow in case of a positive reaction. Different LAMP primers were compared and a newly designed set targeting the gene encoding the tegument protein V67 provided best results, enabling readout within 8-30 minutes. LAMP showed less cross-reactions with other ruminant alphaherpesviruses than qPCR but was 10-fold less sensitive. However, the detection limit for LAMP was as low as 14 copies. The test performance was analyzed using 26 well-characterized BoHV-1 positive and negative nasal swabs from cattle with respiratory disease. All samples were correctly identified when using column-extracted DNA. Using a simple DNA precipitation method, only two weak-positive samples turned indeterminate.

Combining this DNA precipitation with a makeshift water bath heated by an immersion heater allowed successful application of the colorimetric LAMP assay under resource-limited conditions. It can therefore help managing IBR/IPV outbreaks where sophisticated laboratory equipment is unavailable.

Keywords: colorimetric LAMP; bovine alphaherpesvirus 1 (BoHV-1); infectious bovine rhinotracheitis (IBR); rapid detection; resource-limited application

VIRUS GLOSSARY

BoHV-1	Bovine alphaherpesvirus 1
BoHV-1.1	Bovine alphaherpesvirus subtype 1.1
BoHV-1.2a	Bovine alphaherpesvirus subtype 1.2a
BoHV-1.2b	Bovine alphaherpesvirus subtype 1.2b
BoHV-2	Bovine alphaherpesvirus 2
BoHV-5	Bovine alphaherpesvirus 5
BRSV	Bovine respiratory syncytial virus
BuHV-1	Bubaline alphaherpesvirus 1
CvHV-1	Cervid alphaherpesvirus 1
CvHV-2	Cervid alphaherpesvirus 2
PI-3	Parainfluenza virus type 3

SUBMITTED MANUSCRIPT

Rapid and simple colorimetric loop-mediated isothermal amplification (LAMP) assay for the detection of bovine alphaherpesvirus 1

Deborah Peltzer[a,*], Kurt Tobler[a], Cornel Fraefel[a], Madeleine Maley[b], Claudia Bachofen[a]

[a]Institute of Virology, Vetsuisse Faculty, University of Zurich, 8057 Zurich, Switzerland

[b]Moredun Research Institute, Pentlands Science Park, Penicuik, Midlothian EH26 0PZ, UK

[*]Corresponding author.

E-mail address: deborah.peltzer@bluewin.ch (D. Peltzer).

Tel.: +41 78 884 08 90

Highlights

1. Newly designed LAMP primers target the gene of the tegument protein V67

2. The assay delivers results after 8-30 minutes by changing color from pink to yellow

3. New LAMP primers deliver results significantly faster than primers published so far

4. The detection limit of the LAMP assay was found to be 14 copies/µl

5. Simple DNA precipitation permits low-cost application in resource-limited settings

Abstract

As the causative agent of Infectious Bovine Rhinotracheitis (IBR) and Infectious Pustular Vulvovaginitis/Balanoposthitis (IPV/IPB), bovine alphaherpesvirus 1 (BoHV-1) is responsible for high economic losses in the cattle industry worldwide. This study aimed to establish a fast, colorimetric loop-mediated isothermal amplification (LAMP) assay for the detection of viral DNA. Phenol red is used as pH-sensitive readout, relying on a distinct color change from pink to yellow in case of a positive reaction. LAMP reactions with different primers were compared and a newly designed set targeting the gene encoding the tegument protein V67 provided best results, enabling readout within 8-30 min. LAMP showed less cross-reactions with other ruminant alphaherpesviruses than qPCR but was 10-fold less sensitive. However, LAMP still detected down to 14 copies. The test performance was evaluated using 26 well-characterized nasal swabs from cattle with respiratory disease. All samples were correctly identified when using column-extracted DNA. Using a simple DNA precipitation method, only two weak-positive samples turned indeterminate. Combining this DNA precipitation with a makeshift water bath heated by a gastronomic immersion heater allowed successful application of the colorimetric LAMP assay under resource-limited conditions. This technique can therefore help in managing IBR/IPV outbreaks where sophisticated laboratory equipment is unavailable.

Keywords: colorimetric LAMP; isothermal amplification; bovine alphaherpesvirus 1; infectious bovine rhinotracheitis; rapid detection; resource-limited application

1 Introduction

Bovine alphaherpesvirus 1 (BoHV-1) is a highly contagious pathogen responsible for considerable economic losses in the cattle industry worldwide due to a variety of clinical manifestations and imposed trade restrictions. It is the causative agent of Infectious Bovine Rhinotracheitis (IBR), and Infectious Pustular Vulvovaginitis (IPV) / Infectious Pustular Balanoposthitis (IPB).

BoHV-1 is a member of the genus *Varicellovirus* within the *Herpesviridae* family. Three subtypes of BoHV-1 are distinguished. Subtype 1.1 is associated with respiratory disease, while subtypes 1.2a and 1.2b are related to IPV/IPB (Nandi et al., 2009). The viral genome consists of linear double-stranded DNA with a total length of 136 kilo base pairs (kbp) (Schwyzer and Ackermann, 1996).

Upon primary respiratory infection, animals can develop nasal discharge, hyperemia of the nasal mucosa with necrotic foci ("red nose"), cough, pyrexia, apathy and anorexia, often accompanied by a notable milk drop. Disease severity can vary greatly from asymptomatic to severe clinical manifestations, depending on host, viral and environmental factors (Nettleton and Russell, 2017). Abortion can be a sequela of respiratory infection of seronegative cows (Muylkens et al., 2007). During genital infection, animals display frequent micturition and hyperemic genital organ mucosa covered with pustules. Reduced fertility and poor semen quality are possible consequences (Nandi et al., 2009). However, IPV/IPB is less prevalent than IBR, due to wide use of artificial insemination methods and exclusion of infected bulls from artificial insemination centers (Nettleton and Russell, 2017). Following primary infection, the virus establishes latency in the sensory neurons of regional ganglia, e.g. the trigeminal ganglion in the case of IBR, from where it can reactivate under stressful conditions or corticosteroid treatment (Nandi et al., 2009). Latently infected carriers therefore constitute an epidemiologically important reservoir. They pose a constant risk for re-excreting BoHV-1 and infecting other animals, particularly given the fact that re-excretion often occurs asymptomatically (Muylkens et al., 2007). The above-mentioned symptoms and characteristics of the virus have an extensive economic impact on the cattle industry – directly by decreasing the fitness of the animals, as well as indirectly through trade restrictions imposed by the listing of IBR/IPV as notifiable disease in the Terrestrial Animal Health Code (OIE). To date, only a few countries have been able to eradicate BoHV-1, namely Switzerland, Sweden, Norway, Finland, Denmark and Austria (Ackermann and Engels, 2006). In many countries, especially in Europe, national eradication campaigns are ongoing. Otherwise, the virus is still widespread around the globe with varying seroprevalence (Biswas et al., 2013).

Eradication programs and control measures against the spread of IBR mostly rely on antibody detection because it also enables the identification of latently infected animals. There exist specific gE-ELISAs, which allow for herd screening, bulk milk and also individual testing in herds, that were vaccinated with gE-deleted marker vaccines. This allows for a differentiation between vaccinated and naturally infected animals (Nettleton and Russell, 2017). Virus isolation and propagation in cell culture and PCR, on the other hand, are common methods to detect infectious virus and viral DNA, respectively, in acute clinical outbreaks. Although qPCR secured its place as a standard diagnostic procedure for the diagnosis of acute BoHV-1 infections, the duration, the costs and need for special equipment can be hindering factors, particularly under resource-limited circumstances. Simpler, faster and cheaper applications such as loop-mediated isothermal amplification (LAMP) have already been established for an extensive variety of pathogens. The method was developed by Notomi et al. (2000) and was steadily improved (Nagamine et al., 2002; Notomi et al., 2015). LAMP uses a special polymerase with an intrinsic strand displacement activity, which allows for incubation at a constant temperature, therefore, no thermocycler is needed to run the assay. Six primers are used to amplify the target region, making LAMP highly specific. Furthermore, the mechanism of target amplification is extremely efficient, leading to an accumulation of up to 10^9 copies in less than an hour (Notomi, 2000). This massive amplification and the concurrent pH change enable optical readout options such as color change or turbidimetric measurement. Thus, LAMP is an attractive alternative tool in resource-limited areas and in situations where time plays a crucial role in controlling the spread of a disease. Several LAMP assays for the detection of BoHV-1 have already been developed (El-Kholy et al., 2014; Fan et al., 2018; Pawar et al., 2014; Socha et al., 2017). However, these assays mostly rely on fluorescence measurements and gel electrophoresis, which both require further special equipment. In this study we therefore aim to establish a BoHV-1 LAMP assay with a simpler readout, using phenol red as a visual indicator dye. In addition, a fast and easy DNA precipitation method was evaluated, and new primers were designed and tested. The goal was to shorten and simplify the overall procedure - not only of the LAMP reaction itself, but also of the preceding sample preparation - to enable early disease management during outbreaks while also saving resources.

2 Material & Methods

2.1 Samples

2.1.1 Cells and viruses

BoHV-1.1 strain Jura served as positive control for the establishment of this LAMP assay. Strain Jura was originally isolated from an IBR outbreak in the canton of Jura in Switzerland in 1972 (Metzler et al., 1986). Furthermore, two other BoHV-1 strains (BoHV-1.1 strain Cooper (Mayfield et al., 1983) and BoHV-1.2 strain K22 (Kendrick et al., 1958)) and the following bovine and other ruminant alphaherpesviruses were included in the study: bovine alphaherpesvirus 5 (BoHV-5, strain N569 (French, 1962)), bovine alphaherpesvirus 2 (BoHV-2 strain V766), cervid alphaherpesvirus 1 and 2 (CvHV-1 (Inglis et al., 1983), CvHV-2 (Ek-Kommonen et al., 1986)) and bubaline alphaherpesvirus 1 (BuHV-1 (St George and Philpott, 1972)). While BoHV-1 strains Jura, Cooper, and K22, and BoHV-2 and BoHV-5 were specifically passaged for this work, CvHV-1, CvHV-2, and BuHV-1 cell culture supernatant was provided by the diagnostics unit of the Institute of Virology (University of Zurich, Switzerland). All herpesvirus species used in the study underwent a limited number of *in vitro* passages on Madin-Darby bovine kidney (MDBK) cell culture and were harvested when a cytopathic effect (CPE) was visible in 90% of the cells. Cultivation and harvest were carried out according to an in-house protocol, which is based on the guidelines of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2013).

2.1.2 BoHV-1 BAC

A BoHV-1.1 BAC (bacterial artificial chromosome) (strain Jura), previously described by Gabev et al. (2009), was used to compare the analytical sensitivity of the qPCR and the LAMP assay. The DNA concentration of the starting material was measured to be 204 ng/µl using a Qubit™ 2.0 Fluorometer (Life Technologies™, Carlsbad, CA, USA) and the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific,Waltham, MA, USA) according to the manufacturer's instructions. Copy numbers were calculated to be 1,4 $x10^{9}$ copies/µl and were then serially 10-fold diluted down to 1,4 $x10^{-1}$ copies/µl in UTM® nasal swab medium (Copan Italia S.p.A, Brescia, Italy).

2.1.3 Clinical samples

Supernatant from 26 nasal swabs (in Virus Transport Medium) collected from cattle with respiratory disease was kindly provided by the Virus Surveillance Unit (VSU) of the Moredun Research Institute (MRI) in Scotland (Penicuik, UK). The swabs were sent to MRI by different external veterinarians from 2017 to 2019. Twelve nasal swabs tested positive for BoHV-1 by qPCR at the MRI and therefore served as a positive control group. Fourteen nasal swabs that tested negative for BoHV-1 were used as negative controls. As proven by diagnostic evaluation at the MRI (Multiplex-Tandem Bovine Respiratory Diagnostic PCR, AusDiagnostics, Australia), all 26 samples showed co-infection with one or more pathogens involved in the development of bovine respiratory disease (BRD) such as Parainfluenza virus type 3 (PI-3), Bovine Respiratory Syncytial Virus (BRSV), *Pasteurella multocida,* and *Mannheimia haemolytica*. More detailed information on the samples can be found in the supplementary material section (Tab. A). Upon arrival, supernatants were aliquoted and stored at -20°C until further use.

2.2 DNA isolation

2.2.1 QIAamp® DNA Mini Kit

DNA was extracted using the QIAamp® DNA Mini Kit from Qiagen (Düsseldorf, Germany) according to the protocol supplied by the manufacturer. An eluate negative control was included in every extraction series in order to detect contamination during the extraction process. The extracted DNA was eluted in 60 µl of diethylpyrocarbonate (DEPC) treated water and stored at -20°C until further use. BoHV-1 strain Jura DNA extracted by this protocol served as positive control.

2.2.2 Extraction after Vingataramin and Frost (2015)

In the course of adapting the assay to the usage in limitedly equipped laboratories, DNA was also extracted according to a protocol published by Vingataramin and Frost (V&F) in 2015 with minor modifications.

Briefly, 100 µl of nasal swab transport medium were added to 455 µl of extraction solution in a 1.5 ml Eppendorf tube. The extraction solution consisted of Sodium hydroxide (NaOH) (240 mM, final concentration 200 mM), Ethylenediaminetetraacetic acid (EDTA) (2.7 mM, final concentration 2.25 mM) and Ethanol 100% (74%, final concentration 61%). The mixture was incubated at 80°C for 10 min. Afterwards, the mixture was centrifuged at 2,000 x *g* with a portable LLG-uniCFUGE 2 bench-top mini-centrifuge (LLG Labware, Wilmington, DE, USA) for 3 min. The supernatant was thoroughly discarded and the DNA pellet at the bottom of the tube retained. This pellet was re-suspended in 60 µl of DEPC treated water and stored at -20°C until further use.

2.3 BoHV-1 and 12S qPCR

A qPCR targeting the gB coding region of BoHV-1 was carried out as reference method. The assay for BoHV-1 was performed according to Abril et al. (2004) with minor modifications in the template volume. The qPCR was run at a final volume of 25 µl per reaction, consisting of 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems, Waltham, MA, USA), 240 nM of forward (5'-TGTGGACCTAAACCTCACGGT-3') and reverse (5'-GTAGTCGAGCAGACCCGTGTC-3') primer, 160 nM of the probe (5'-FAM-AGGACCGCGAGTTCTTGCCGC-TAMRA-3'), 8.9 µl DEPC treated water and 2 µl template.

As an internal control to verify successful DNA extraction, a qPCR amplifying a part of the reference gene encoding for 12S mitochondrial rRNA was used (Stahel et al. 2013). Primer and probe design were based on a consensus sequence between previously published 12S rDNA sequences of various bovid taxa (Gatesy et al., 1997; Stahel et al., 2013). The final reaction mixture of 25 µl for the 12S qPCR contained 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems, Waltham, MA, USA), 600 nM of forward (5'-GCGGTGCTTTATAYCCTTCTAGAG-3') and reverse (5'-TTAGCAAGRATTGGTGAGGTTTATC-3') primer, 160 nM of the probe (5'-VIC-AGCCTGTTCTATAAYCGAT-MGBNFQ-3'), 8.9 µl DEPC treated water and 2 µl template.

The PCR reactions were run on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) and the cycling conditions for both qPCR assays corresponded to those published by Abril et al. (2004). The threshold value was set manually

at 0.1 for BoHV-1 and at 0.05 for 12S. The result was considered positive if the fluorescence signal exceeded the threshold value and exponential amplification was observed.

2.4 LAMP primer design

Seven primer sets were compared at the beginning of this study. The sets targeting the genes encoding for gB (Fan et al., 2018), gC (Pawar et al., 2014), gD, and gE (Socha et al., 2017) were already published (Suppl. Tab. B). Additionally, three sets targeting the sequences of the catalytic subunit of the Polymerase (Pol), the Helicase (Hel), and the tegument protein V67 (V67) (Tab. 1) were designed using the LAMP designer 1.16 software from Premier Biosoft (Palo Alto, CA, USA). These three regions were chosen because of their relatively low GC content (60.2%, 61.8% and 61.8% respectively). Regions of low GC-content were identified in the NCBI reference strain sequence of BoHV-1 (GenBank accession number NC_001847.1 with the GeneQuest application of the DNASTAR Lasergene V.17 software (DNASTAR, Madison, WI, USA). This reference strain was also used for primer design, with primers designed within the above regions.

Table 1

Sequences and final concentrations of the newly designed LAMP primers used for the final assay

Target gene Genome position[1]	**Primer**	**Sequence**	**Final Concentrations**
V67 [U_S 1.67] 114'483 – 114'731	F3	5'-GTACTGGCTCATGTTTCCC-3'	0.2 µM
	B3	5'-CTGCTGGTGAAAGTTCCC-3'	0.2 µM
	FIP	5'-TCGTTAAGCTTCCGCACAACTGTTGAGGTAGAAGCGGTC-3'	1.6 µM
	BIP	5'-TGTTCCGTCGTAAAGCTGACGGTTCACTTTGAATGTGTTCCC-3'	1.6 µM
	LF	5'-AGACGAGTGCTACGAGGA-3'	0.4 µM
	LB	5'-AAAGAACTGCAGCGGTCG-3'	0.4 µM

[1]*The genome position refers to the position of the LAMP amplicon and is relative to the reference sequence for BoHV-1 (GenBank accession number NC_001847.1).*

F3 = outer forward primer; B3 = outer backward primer; FIP = forward inner primer; BIP = backward inner primer; LF = forward loop primer; LB = backward loop primer; V67 = tegument protein V67; U_S = unique short sequence. The sequences of the Pol and Hel primer sets are only provided in Suppl. Tab. B as they proved inferior to V67.

2.5 LAMP assay

In a first stage, the real-time WarmStart® LAMP Kit (New England Biolabs, Ipswich, MA, USA), which includes a master mix and a separate tube of fluorescent dye for readout in a qPCR machine, was used for the optimization of the LAMP assay. The real-time measurement allowed for a precise representation and evaluation of amplification and melt curve data during the establishment of the assay. The Colorimetric WarmStart® LAMP 2x Master Mix (New England Biolabs, Ipswich, MA, USA) was used as a visual assay. The Colorimetric WarmStart® LAMP 2x Master Mix contains the same components (except for a low-Tris reaction buffer and additional phenol red) as the master mix of the real-time WarmStart® LAMP Kit. Because the Colorimetric Master Mix does not come with a fluorescent dye, but a precise representation of amplification and melt curve to control the visual readout during the second stage of the assay establishment was still desired, Syto™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Carlsbad, CA, USA) was added to the Colorimetric Master Mix. This allowed the concurrent evaluation of two readouts (real-time fluorescence and end-point visual color change). Syto™ 9 was chosen due to the results of a study previously published by Oscorbin et al. (2016), showing less inhibitory effects and a higher signal-to-noise ratio compared to other intercalating fluorescent dyes. The performance of Syto™ 9 and the fluorescent dye of the real-time WarmStart® LAMP Kit was compared beforehand in a separate experiment and was identical for both dyes (data not shown).

The final reaction volume of 12.5 µl for the real-time WarmStart® LAMP Kit consisted of 6.25 µl WarmStart® LAMP 2x Master Mix, 0.25 µl LAMP Fluorescent Dye (50x), 1.25 µl 10x primer mix, 3.75 µl DEPC treated water and 1 µl of template.

The final reaction volume of 12.5 µl for the Colorimetric WarmStart® LAMP Master Mix consisted of 6.25 µl Colorimetric WarmStart® LAMP 2x Master Mix, 0.5 µl 25x Syto™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Carlsbad, CA, USA), 1.25 µl 10x primer mix, 3.5 µl DEPC treated water and 1 µl of template. Syto™ 9 Green Fluorescent Nucleic Acid Stain was prepared as a 25x stock solution according to Tanner and Evans (2013). The template was denatured at 95°C for 5 min and immediately placed on ice before being added to the reaction mix.

The reaction mix was then incubated at 65°C for 60 min. Afterwards, a melt curve consisting of the following steps was added: 1.6°C/sec up to 95°C, 15 sec at 95°C, 1.6°C/sec down to 60°C, 1 min at 60°C, 0.1°C/sec up to 95°C and finally 15 sec at 95°C. A QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) was used to run the assay. For

the analysis of the results and information on the fluorescence measurement, please refer to chapter 2.6.

2.6 Detection of LAMP products

Two readout methods were used in this work. First, a real-time fluorescence measurement was used throughout the entire study. Second, a visual readout based on phenol red was employed in a second stage, once the assay was optimized.

Regarding the real-time readout, fluorescence emission of the fluorescent dye from the real-time WarmStart® LAMP Kit and of the Syto™ 9 was measured in the SYBR channel. A reaction was considered positive when meeting the following criteria: the signal exceeded the manually set fixed threshold value (beginning of stable exponential amplification) of 16,480, it amplified exponentially, and the melt curve analysis showed a peak at around 89°C. The time point where the fluorescence signal crosses the threshold is called time to reaction (TTR) or reaction time. Reactions meeting the following criteria were considered negative: no measurement of an exponential amplification curve, and appearance of a flat line in the melt curve analysis. Reactions with an amplification curve exceeding the threshold, but with a melt curve peak at a lower temperature than 89°C or with multiple melt curve peaks, were considered false positive due to non-specific amplification.

Phenol red is included in the Colorimetric WarmStart® LAMP 2x Master Mix as a visual indicator dye. Due to a drop in pH caused by an accumulation of protons during a positive LAMP reaction, the dye changes color from pink to yellow (Tanner et al., 2015). In the case of a negative reaction, the color remains pink. It is important to note that the initial pH of the template plays a vital role in the initial color of the reaction mix. If an acidic template is added, the starting color becomes more reddish than pink and must not be classified as a questionable positive result.

2.7 Optimization of LAMP conditions

The effect of different concentrations of Betaine (0M – 1.6M), DMSO (0% - 7.5%), additional $MgSO_4$ (0mM – 2mM), or additional dNTPs (0mM – 0.4mM) in the reaction mix was analyzed. Also, the effect of doubling the concentration of FIP/BIP primer and doubling the concentration

of all primers was evaluated. Further, the effect of using different volumes of template (1 – 5 µl) was analyzed.
Lastly, a temperature gradient (61°C – 70°C) was carried out to determine the optimal incubation temperature.

2.8 Analytical specificity and sensitivity

Extracted DNA of the ruminant alphaherpesviruses was first diluted to approximately the same Cq value (ΔCq 24,4 ± 0.8). In a next step, the diluted samples were analyzed by LAMP by means of real-time fluorescence measurement and concurrent visual detection at the end of incubation. Results were then compared to those obtained by qPCR.
To evaluate the analytical sensitivity, the minimum detectable copy number was determined by simultaneously testing the serially diluted BoHV-1 BAC (see chapter 2.1.2) by BoHV-1 qPCR, and concurrent visual and qLAMP.

2.9 Testing of clinical samples

A total of 26 nasal swabs supernatants kindly provided by the Moredun Research Institute (Penicuik, UK) were used to test the performance of the newly established LAMP assay with field samples. The samples were first used untreated, i.e. no DNA extraction step was included, only the denaturation of the nasal swab supernatant at 95°C for 5 min was performed. Alternatively, DNA was extracted using the QIAmp® DNA Mini Kit (Qiagen, Düsseldorf, Germany) (for details see chapter 2.2.1) and applying the method described by Vingataramin and Frost (for details see chapter 2.2.2). Finally, the untreated nasal swabs, as well as the extracted DNA, were tested by LAMP and qPCR. Where sufficient nasal swab supernatant was available, extractions were performed in duplicate to ensure biological replicability. Each extraction series was tested twice by qPCR and twice by LAMP to ensure technical replicability.

2.10 Adaptation to resource-limited settings

A goal of this study was to use as little equipment and time for the performance of the assay as possible, to make the test applicable in situations where only limited laboratory facilities are available. Therefore, the following set up was assessed. A commercial gastronomic immersion heater for low-temperature long-time cooking of vacuumed goods, a so-called sous-vide stick (Aicok Sous Vide SV-8001), was placed in an insulating rubber ice bucket containing water. The sous-vide stick has ability to heat water up to 99°C and maintain the temperature with an accuracy of ± 0.5°C. This heated water bath was used for DNA precipitation, DNA denaturation and LAMP incubation. To ensure consistency of temperature everywhere in the water bath, temperatures (65, 80 and 95°C) were controlled at different spatial points with a TFN 520 thermometer (ebro GmbH, Urdorf, Switzerland). A bench-top mini-centrifuge was used for the preparation of the LAMP reaction mix and the DNA extraction. Apart from these two tools, pipets and pipet tips, 0.1 ml PCR tubes, 1.5 ml Eppendorf tubes, racks that fit and fix the different tubes for incubation in the water bath, a rack to prepare the DNA precipitation and the LAMP reaction mix on the bench, a frozen cold pack, and a weight to submerge the rack for the LAMP incubation were used. With this equipment, all the necessary steps could be performed, which included the DNA precipitation after V&F, denaturation of the DNA and the LAMP assay. For the detailed and illustrated protocol see Suppl. Fig. A.

2.11 Statistical analyses

To compare the TTR results of different primer sets and of TTR between different incubation temperatures, the NCSS 10 statistical software (NCSS, LLC; Utah, USA) package was used. For overall comparison, the Kruskal-Wallis One-Way ANOVA on Ranks and for pair-wise comparison the paired t-test were applied. P values below 0.05 were regarded as statistically significant.

3 Results

3.1 Comparison of different extraction methods

Two extraction methods were used in this study, namely a commercial, column-based extraction kit (QIAmp® DNA Mini Kit) and the DNA precipitation described by Vingataramin and Frost (Vingataramin and Frost, 2015). Figure 1 illustrates and compares the extraction efficiency of both approaches. No Cq values are displayed for undiluted V&F DNA because remaining traces of NaOH buffer inhibited amplification in qPCR. Cq values were in average 2.8 cycles lower with the column-extracted DNA. The difference was in some samples only 1-2 cycles (sample 20 or 21), in others up to 5 cycles (sample 19). All positive samples however were still identified as such. Negative samples were also compared, but as no false positive or unspecific reactions were observed, these were not included in the diagram.

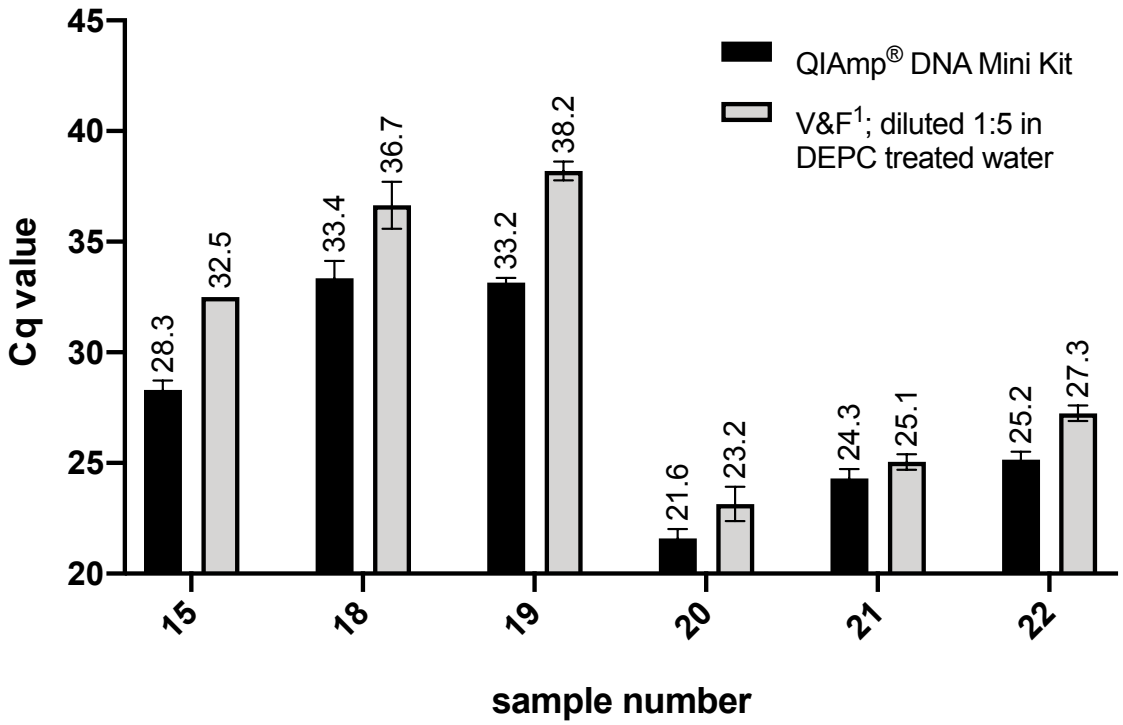

Figure 1: *Comparison of DNA extracted with the QIAmp® DNA Mini Kit and precipitated according to [1]Vingataramin and Frost (Vingataramin and Frost, 2015) diluted 1:5 in DEPC treated water. Extraction efficiency was controlled by 12S and BoHV-1 qPCR (data for 12S not shown). Six positive samples with sufficient nasal swab medium for concurrent extraction by both methods are displayed. For three more positive samples volumes were insufficient and they are therefore missing in this figure. Sample numbers match sample numbers in figure 6 and 7. Extraction and precipitation were carried out simultaneously from the same vial. Data are shown as mean ± SD of two extractions analyzed in duplicates.*

3.2 LAMP Primer

Seven primer sets were tested for their reaction time to determine the most efficient set (Fig. 2) using DNA extracted from BoHV-1 (strain Jura) positive cell culture supernatant (~ Cq value 18). The newly designed primers targeting the gene for the tegument protein V67 showed a significantly faster TTR (~ 8.3 min; $p < 0.000001$) compared to the second best primer set tested (Pol, ~ 13.5 min) and were therefore used for all following experiments. All other sets had far longer TTRs. The primers targeting the gene encoding gB needed the longest time for the fluorescence signal to cross the threshold (~ 44 min).

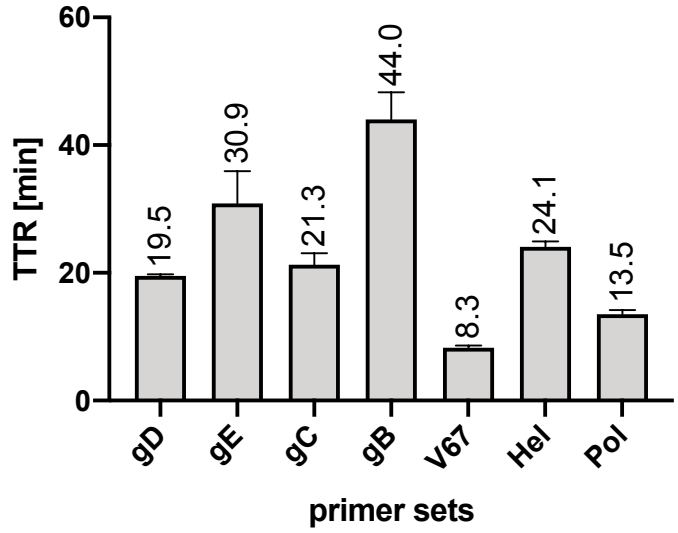

Figure 2: *Comparison of different primer sets. The experiment was run twice and in triplicate for each primer set. Data are shown as mean ± SD. V67: tegument protein V67; Hel: Helicase; Pol: Polymerase.*

3.3 Optimization of LAMP conditions

Different conditions were evaluated to optimize the qLAMP assay using the real-time WarmStart® LAMP Kit. The addition of Betaine, DMSO, Betaine and DMSO combined, $MgSO_4$, dNTPs or higher primer concentrations to the reaction mix did not reduce the reaction time (data not shown). The master mixes of the WarmStart® kits were therefore used as recommended by the manufacturer. Since increasing the template volume did not have any effect on the reaction time either (data not shown), it was decided to use 1 µl of template in a total reaction volume of 12.5 µl. The temperature gradient revealed the optimal incubation

temperature to be 65°C, as suggested by the instructions of the WarmStart® LAMP Kit manual (Fig. 3).

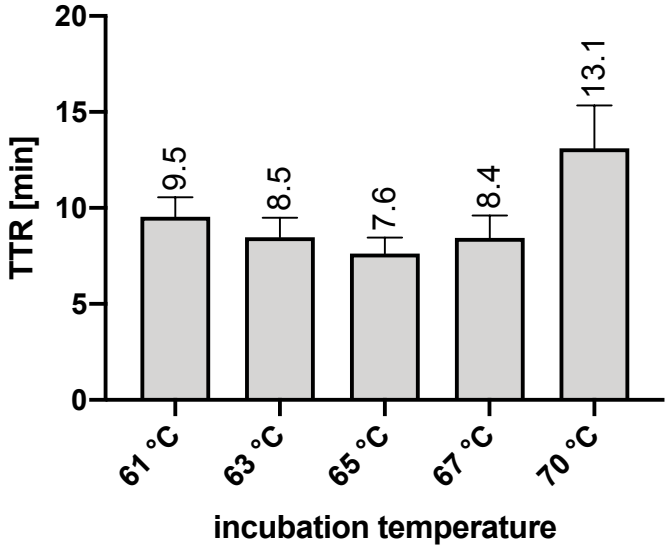

Figure 3: *Temperature gradient showing the effect of different incubation temperatures on the TTR. The experiment was run twice and in triplicate for each primer set. Data are shown as mean ± SD. The TTR was significantly lower at 65°C compared to the other temperatures (p = 0.002 – 0.00001).*

3.4 Analytical specificity

The analytical specificity was determined by testing various bovine and other ruminant alphaherpesviruses by qPCR, and by visual and concurrent qLAMP using the Colorimetric WarmStart® LAMP master mix with an added fluorescent dye (Fig. 4). All DNA samples were diluted to approximately the same Cq values of 24-25. While the BoHV-1 qPCR cross-reacted with all examined viruses except for BoHV-2, the LAMP assay only amplified the different BoHV-1 strains as well as CvHV-2 and BuHV-1. However, the TTR for BoHV-1 strains was much lower, ranging from 8.6 to 16.9 min and leading to a clear color change from pink to yellow. CvHV-2 and BuHV-1 only gave a positive result in qLAMP readout after 42 and 56.4 min respectively, but the colorimetric readout remained indeterminate. Only when using undiluted BuHV-1 (~Cq value of 18.2) a cross-reaction occurred after 27.7 min resulting also in a color change to yellow (data not shown). Long reaction times are usually suspicious for non-specific amplification (e.g. primer dimer). Melt curve analysis however revealed the specificity of the reactions. BoHV-5 did only cross-react at ~ 33.8 min resulting in color change when used at the original concentration (~ Cq value of 18.2) instead of the diluted template (~

Cq value of 25), as did CvHV-1 (~ Cq value of 18.2 equaled a TTR of ~ 50.9 min, but no color change) (data not shown).

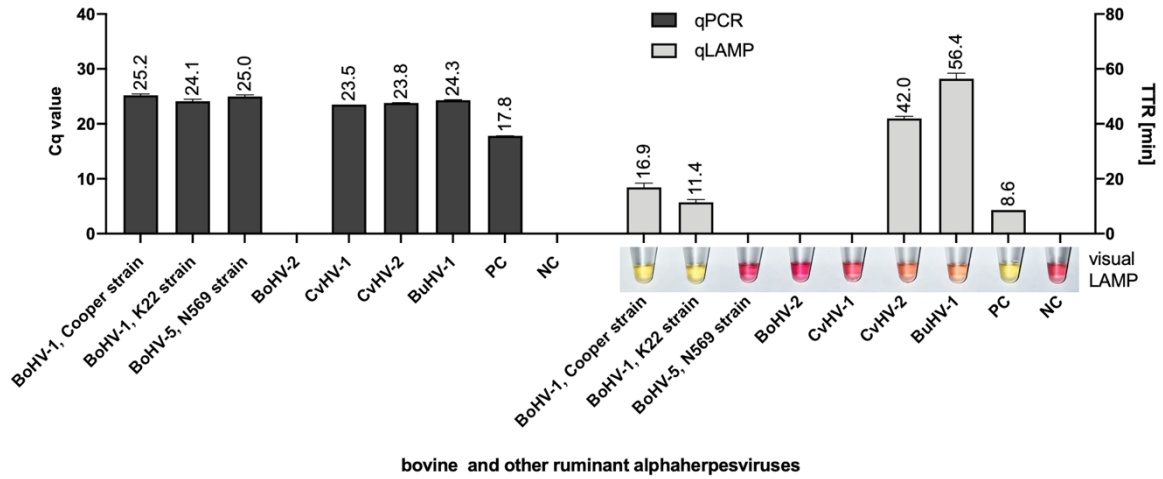

Figure 4: *Cross-reactivity of the BoHV-1 qPCR and the qLAMP assay with different bovine and ruminant alphaherpesviruses. Below the graph on the right side, the corresponding results of the visual readout are added. The experiment was run twice and in duplicates. Data are shown as mean ± SD. PC: positive control (BoHV-1.1 strain Jura); NC: negative control (DEPC treated water).*

3.5 Analytical sensitivity

The sensitivity of the assay was evaluated using a serially tenfold diluted BoHV-1.1 BAC (strain Jura) ranging from 1.4 x 10^5 to 1.4 x 10^{-1} copies/µl. The dilution series was tested by qPCR, and by visual and concurrent qLAMP (Fig. 5). While the qPCR detected as few as 1.4 x 10^0 DNA copies, the detection limit of the LAMP assay was 1.4 x 10^1 DNA copies. Cq values of the qPCR ranged from 21 for the highest, to 37.5 for the lowest detectable copy number. TTRs ranged from 12.6 to 26.9 min respectively. Thus, the LAMP assay is slightly less sensitive than the qPCR, detecting ten times less copies.

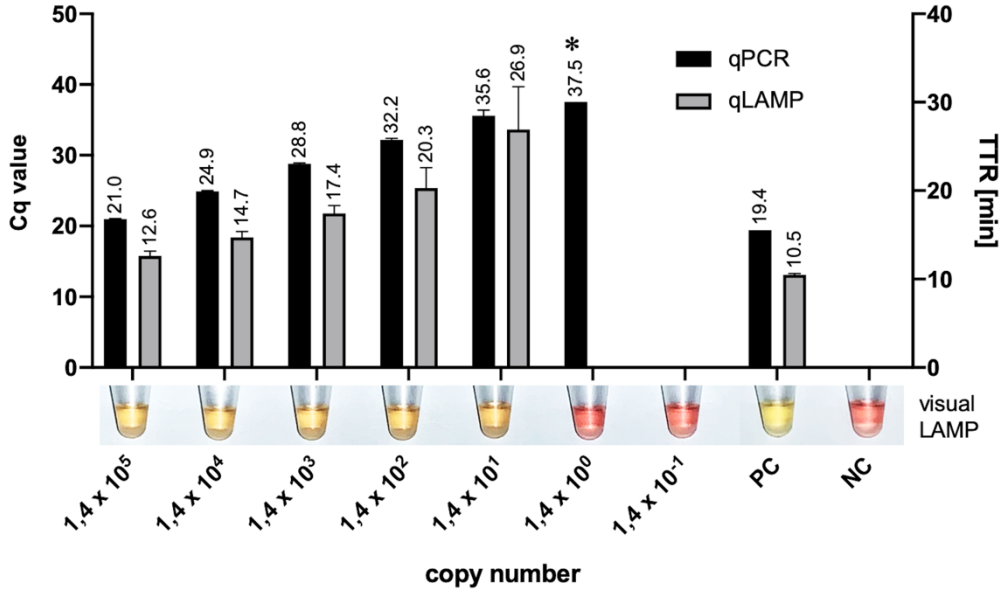

Figure 5: *Data are shown as mean ± SD. qPCR as "gold standard" was run once in duplicates, the LAMP assay was performed twice and in duplicates.* ✻ *represents a single value because one reaction of the duplicate remained negative. PC: positive control (BoHV-1.1 strain Jura); NC: negative control (DEPC treated water).*

3.6 Testing of clinical samples

Twenty-six nasal swabs supernatant from clinical cases of bovine respiratory disease were tested for the presence of BoHV-1 DNA by qPCR and by visual and qLAMP (Fig. 6). Initial attempts of using the nasal swab supernatant directly without DNA extraction did not yield satisfactory results due to many false negative and false positive results (data not shown).

When extracting DNA using the QIAmp® DNA Mini Kit, the results of the qPCR and of the visual and concurrent qLAMP were identical (Fig.6, uppermost row of three). Samples 16, 17 and 27 originally tested positive for BoHV-1 at the MRI with Cq values of around 40, 40 and 32, respectively, but always tested negative in qPCR at our lab. Since this may be due to DNA degradation by freeze-thawing or shipment, we used our qPCR results to determine the status of the samples. With DNA precipitated by the method of V&F on the other hand, an inconsistency of results could be observed (Fig.6, middle row of three). The qPCR gave false-negative results in some replicates of 5 positive samples. Interestingly, the qLAMP was less affected, as all positive samples were detected, also when using DNA extracted by columns. The visual LAMP however, delivered variable results and color change could not be clearly judged at times.

When diluting V&F DNA 1:5 in DEPC treated water prior to denaturation and addition to the reaction mix, results became more consistent (Fig.6, bottom row of three). All samples apart from number 18 and 19 gave the same result as samples extracted with the QIAmp® DNA Mini Kit. Sample 18 had a mean Cq value of 33.4 when extracted with the QIAmp® DNA Mini Kit, and a Cq value of 36.7 when extracted after V&F and diluted 1:5 in DEPC treated water; sample 19 had Cq values of 33.2 and 38.2 respectively (Fig. 1). The detection limit of LAMP was estimated to equal approximately a Cq value of 37 (Fig. 5). While the number of tested samples is too small for reliable calculation of test performance, the diagnostic sensitivity for these 26 samples using the precipitated DNA diluted 1:5 and visual LAMP was 78% and the diagnostic specificity 100%. All the positive samples (except for one replicate of sample number 18) reacted in less than 30 min. The positive predictive value for the 26 samples was calculated to be 100% and the negative predictive value 89% (Trevethan, 2017).These values were calculated using the qPCR results with DNA extracted by QIAmp® DNA Mini Kit to determine the true status of the samples. The results of samples 18 and 19 were inconsistent in visual LAMP but more replicates were negative than positive and therefore the samples were considered negative for the calculations.

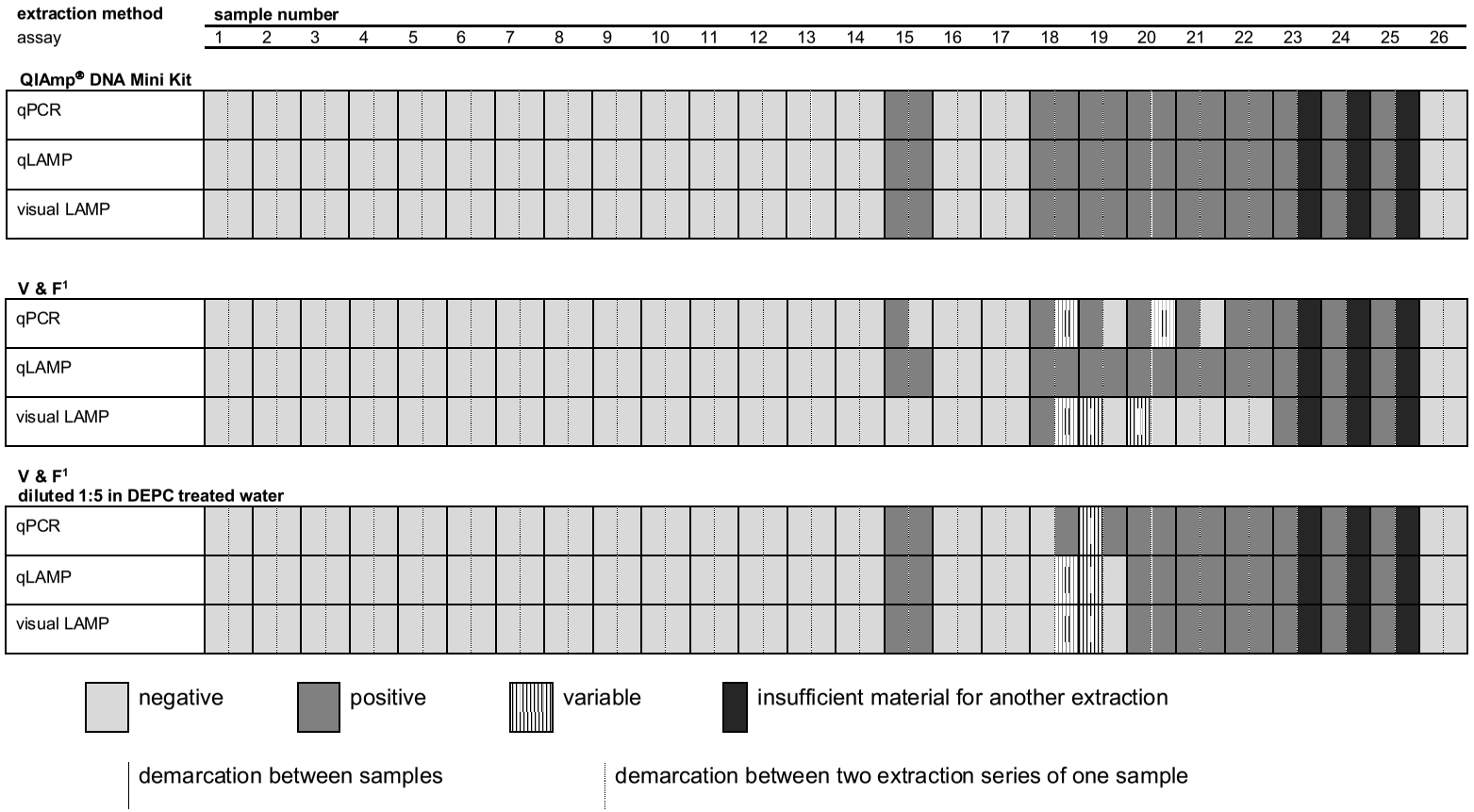

Figure 6: *Overview of the results of the clinical samples tested (n=26). DNA of the samples was extracted and processed in three different manners: (i) with the standardized DNA extraction method using the QIAmp® DNA Mini Kit (uppermost row of three); (ii) according to the protocol of Vingataramin and Frost (middle row of three); (iii) as in (ii) but diluting the eluted DNA 1:5 in DEPC treated water (bottom row of three). For clarity of presentation, a ternary depiction of the results was chosen. With each of the two extraction series, two independent experiments were carried out in duplicates (LAMP) or single assay (PCR). variable: Within the total of four replicates, replicates were not always negative or positive.*

[1]*Extraction method after Vingataramin and Frost, 2015.*

3.7 Adaptation to limited equipment

DNA was extracted from six of the clinical samples with sufficient material left and tested by visual LAMP using only the equipment listed in chapter 2.10 to imitate the use of the assay under limited equipment conditions. The core of the experimental set up consisted of a portable mini-centrifuge (Fig. 7 A) and a water bath, which could be heated to different temperatures with a sous-vide stick (Fig. 7 B). Measuring the temperature in the water bath with an independent thermometer proved consistency and correctness of temperature (± 0.3 °C), also at points that were the farthest away from the heat source. An extraction control consisting of BoHV-1 (strain Jura) cell culture supernatant was added to prove successful DNA extraction. After the centrifugation step, a pellet was not clearly visible in two of the six samples. In these cases, the extraction solution was decanted carefully and possible visible traces of extracted DNA eluted in DEPC treated water. A color change became apparent in some of the samples already after 15 min of LAMP incubation. After 30 min however, a color change was clearly visible in all positive samples and results were identical to those obtained by qLAMP and qPCR. Incubation time may therefore be reduced to 30 or 40 min instead of the 60 min used previously. For better visibility of the color change, a double volume of reaction mix (23 µl) and template (2 µl) were used for the LAMP assay in this experiment. For illustration of the exact set up, and single steps of DNA precipitation and LAMP incubation, a picture series with descriptions is provided in Suppl. Fig. A.

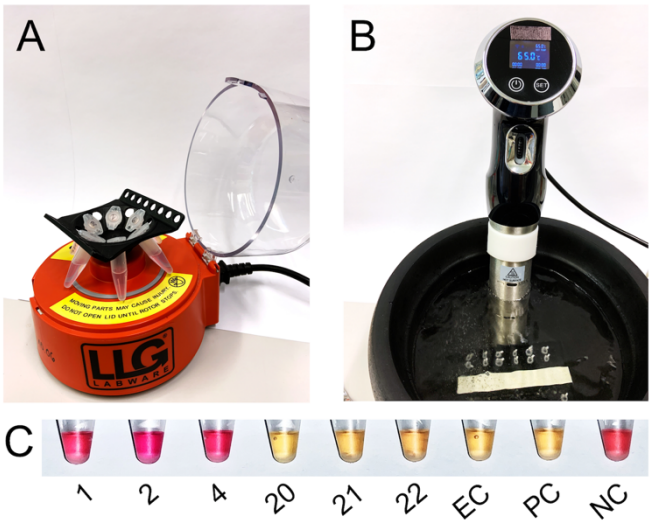

Figure 7: *Adaption to limited equipment. A: mini-centrifuge used for DNA precipitation and preparation of the LAMP reaction mix; B: Sous-vide stick heating water to different temperatures in a rubber ice bucket; C: Results of 6 samples to test this simple set up and only relying on visual readout. Sample numbers match numbers in figures 1 and 6. Samples 1, 2 and 4 were negative for BoHV-1, samples 20, 21 and 22 positive.*
EC: positive extraction control; PC: positive control (BoHV-1 strain Jura; NC: negative control (DEPC treated water).

4 Discussion

Due to its simplicity, speed and the dispensability of sophisticated laboratory equipment, LAMP has become a popular tool for point-of-care diagnostics and has proven particularly useful in situations where no well-equipped lab is at hand such as for diagnosis of leishmaniosis in rural Brazil (Celeste et al., 2019).

BoHV-1 is still widely spread causing significant economic losses to cattle industries worldwide. Affected regions, especially resource-limited areas and regions lacking functioning infrastructure, would profit from an easy, fast, and cost-efficient diagnostic tool.

Here, we report the design of new LAMP primers for the detection of BoHV-1, showing significantly faster reaction times than that of previously published primer sets (Fan et al., 2018; Pawar et al., 2014; Socha et al., 2017) (Fig. 2). The previously published gB primer set showed the longest reaction time (44 min). This relatively weak performance may be explained by the absence of loop primers in the gB primer set, while the other sets include loop primers. Even though not essential for LAMP, loop primers have been shown to accelerate the LAMP reaction greatly (Nagamine et al., 2002). While amplicons obtained with our primers show lower percentage of GC compared to previously published assays, there is no difference in GC content of amplicons between V67, Hel and Pol primers that could explain the significantly better performance of V67 primers (Suppl. Tab. B). Thus, the GC content seems not to be the only influencing factor even though high GC content is known to hinder efficiency of amplification assays (Kool, 2001). Since the BoHV-1 genome has an exceptionally high GC content of 70% (Thiry et al., 2006), choosing the V67, Hel and Pol regions was thought to facilitate strand displacement and therefore enhance amplification and reduce reaction time. However, melting temperature, primer secondary structures and stability at the end of the primers can also influence the efficacy of a primer set.

To our best knowledge, this is the first LAMP assay for the detection of BoHV-1 which uses phenol red as a readout. This indicator dye is already included in the Colorimetric WarmStart® LAMP master mix. Previous studies mainly focused on the usage of hydroxynaphthol blue (HNB) (Goto et al., 2009; Pawar et al., 2014) and SYBR Green I stain or gel electrophoresis (El-Kholy et al., 2014; Pawar et al., 2014; Socha et al., 2017) as visual readout methods. These methods, however, require additional time and consumables such as preparation of the dyes, the preparation of an agarose gel and time to run the electrophoresis. Furthermore, the color change of HNB from purple to sky blue is sometimes difficult to discern by eye and adding SYBR Green stain I post incubation poses a considerable risk to contaminate surfaces or

equipment when opening the tubes containing massively amplified LAMP products. Moreover, the color change of phenol red from pink to yellow can be clearly distinguished. A further advantage of this master mix is the possibility of a set up at room temperature – a helpful feature for field applications. Yet a disadvantage lies within the pH-sensitivity of this indicator dye. When using nasal swab supernatant directly or eluting DNA in anything else than water, the pH of the buffer can have an effect on the starting pH and therefore the color of the reaction mix. This can complicate or even render a visual readout impossible. The manufacturer of the Colorimetric Kit therefore advises not to use more than 10% template volume compared to the final reaction volume to reduce the impact of the pH on the color change. Thus, only 1 µl of template per 12.5 µl reaction was used.

The assay successfully detected all three tested BoHV-1 strains. Cross-reactivity with BuHV-1 and CvHV-2 was observed at very late stages of incubation (Fig. 4) and with BoHV-5 and CvHV-1 only if copy numbers were relatively high (~ Cq values of 18, data not shown). One could therefore argue that the risk of cross-reactions rises with increased titers of shed virus. We aimed at comparing all viruses by LAMP at approximately the same Cq value. While it would have been informative to do so also at Cq values below 20, the copy numbers of several viruses were relatively low (e.g. CvHV-2), even after additional cell culture passages, resulting in Cq values no lower than 25. Subsequently, all viruses were compared at Cq values of 24-25 (Fig. 4). BuHV-1 shows 86% nucleotide identity with BoHV-1 in the V67 target region and is therefore closely related to BoHV-1, explaining the cross-reaction. Interestingly, CvHV-2 that shows the stronger cross-reactivity, i.e. shorter TTR (Fig. 4), is only 77% identical to BoHV-1 V67; moreover the target sequence of BoHV-5 shares 83% nucleotide identity with BoHV-1 but shows less cross-reactivity, as does CvHV-1 with a nucleotide identity with BoHV-1 of 82%. Finally, no cross-reaction was observed with BoHV-2. This is not unexpected as BoHV-2 was the only tested virus belonging to the genus *Simplexvirus* while all other viruses in the study belong to the genus *Varicellovirus* and are therefore more closely related to BoHV-1. Yet it was important to check for this cross-reactivity since serological cross-reactions between these two viruses have been reported (Böttcher et al., 2012) and BoHV-2 occurs with regionally high prevalence (Barnard, 1997; Geiger et al., 1990; Martin et al., 1987). While being excreted primarily through dermal lesions on the udder, contamination of a nasal swab with BoHV-2 cannot be excluded. Since the TTR for BoHV-1 was well below 40 min even with low copy numbers (Fig. 5), lowering the incubation time from 60 to 40 min would be an effective measure to reduce the risk of above-mentioned cross-reactions. Nevertheless, inter-species transmissions of related alphaherpesviruses to cattle do not seem to play an important role in

BoHV-1 epidemiology (Thiry et al., 2006) and false positive results due to infection with other ruminant herpesviruses may therefore be rather unlikely. However, if such an infection is suspected, additional, confirmative analyses are recommended. Previous reports of naturally occurring homologous recombination between BoHV-1 / BoHV-5 (Maidana et al., 2017), and vaccine / wild-type BoHV-1 (d'Offay et al., 2019) may represent a more significant challenge. Therefore, in regions where vaccines are administered frequently and/or where BoHV-1 and BoHV-5 occur simultaneously, a secondary test to identify the virus, sequencing of the LAMP product or probably restriction enzyme analysis (El-Kholy et al., 2014) may be necessary.

The analytical sensitivity of the colorimetric LAMP assay was slightly lower than that of the qPCR, however LAMP still detected 14 copies/µl equaling to a Cq value of 35.6 (Fig. 5). In case of clinically overt IBR outbreaks, animals are typically shedding high quantities of viral particles (Muylkens et al., 2007; Nettleton and Russell, 2017), therefore, a slightly decreased sensitivity may be acceptable for tests deployed in this scenario. However, for surveillance programs where an eradication of IBR is required, the suitability of the LAMP assay should first be thoroughly evaluated.

When testing column-extracted DNA from 26 clinical nasal swabs, our LAMP assay performed equally well as the qPCR and a diagnostic sensitivity of 100% was reached (Fig. 6). The unsatisfactory results obtained by testing supernatant without DNA extraction, might be explained by inhibition due to contaminants, such as humic acid or complex polysaccharids originating e.g. from animal feed or bedding, or unsuitable swab medium. We observed that for example media containing protein denaturizing reagents such as eNAT™ (Copan, Italy) abolish the reaction completely (data not shown). Using DNA extracted after V&F without further dilution also proved unsuitable due to traces of NaOH from the extraction solution inhibiting the qPCR and influencing the pH of the LAMP reaction, thereby disabling visual read out – even if amplification occurred (Fig. 6). A 1:5 dilution of the DNA in water abolished the NaOH-related problems but diagnostic sensitivity decreased to 78% due to two weak positive samples falling below the detection limit of the assay. This may be due to the V&F precipitation being generally somewhat less efficient than the column-based extraction (Fig. 1) and/or a stronger influence of impurities of the nasal swabs (e.g. amount of nasal mucus, purulent material, abraded skin particles) on precipitation. In short, detection of DNA precipitated after V&F and diluted 1:5 in DEPC water is somewhat less sensitive than DNA extracted with the QIAmp® DNA Mini Kit, however the speed, the costs and the limited equipment needed may compensate for the decreased sensitivity in specific circumstances such as field settings or the lack of a well-equipped laboratory. Therefore, we tested the applicability of our LAMP assay under

"field conditions". A simple set up using an insulated rubber ice bucket and a commercial gastronomical immersion heater to form a makeshift water bath and a portable mini-centrifuge to precipitate the DNA proved sufficient for the DNA precipitation after V&F and the visual LAMP assay (Fig. 7, Suppl. Fig. A), offering a cost saving alternative to qPCR and column-based DNA extraction.

Due to the absence of IBR in Switzerland, obtaining positive clinical samples was difficult. Twenty-six supernatants from nasal swabs, kindly provided by the VSU of the MRI (Penicuik, UK), where tested to this end. Based only on these few samples we cannot completely and reliably assess the diagnostic sensitivity and specificity of the assay. However, the samples were known to be also positive for several other pathogens of the bovine respiratory tract such as paramyxoviruses and *Pasteurella multocida* and it was important to proof absence of cross-reaction or non-specific reaction in cases of such mixed infections.

In conclusion, a fast, colorimetric LAMP assay for the detection of BoHV-1 was developed. Analytical specificity appears to be higher than that of the qPCR used as a reference method in this study, but the LAMP assay is around tenfold less sensitive. Compared to other qPCRs however, analytical sensitivity and specificity may differ.

The LAMP assay may be flexibly applied in different settings. Using the real-time readout option and extracted DNA it represents a fast (30 min run time) and cost-efficient alternative to qPCR in routine veterinary diagnostic labs. On the other hand, while sensitivity is slightly reduced, the use of a simple and fast DNA precipitation (Vingataramin and Frost, 2015), portable equipment and the visual readout option (Fig. 7, and Suppl. Fig. A) enables application of the assay as an affordable and practicable diagnostic tool in resource-limited settings.

To reliably assess the diagnostic sensitivity and specificity of the assay, a larger number of clinical samples needs to be tested which is ideally done in a country where, unlike Switzerland, BoHV-1 is still circulating. Also, further studies could focus on whether the assay is applicable to other specimen than nasal swabs, e.g. semen, abortion material or fetal tissue.

Acknowledgements

We thank the Virus Surveillance Unit of the Moredun Research Institute in Scotland (Penicuik, UK) for providing samples from clinical cases. We would also like to thank Mathias Ackermann for his valuable inputs and advice throughout the study. This work was supported by the University of Zürich and the Federal Food Safety and Veterinary Office (national grant number 1.18.03).

References

Abril, C., Engels, M., Liman, A., Hilbe, M., Albini, S., Franchini, M., Suter, M., Ackermann, M., 2004. Both Viral and Host Factors Contribute to Neurovirulence of Bovine Herpesviruses 1 and 5 in Interferon Receptor-Deficient Mice. J. Virol. https://doi.org/10.1128/jvi.78.7.3644-3653.2004

Ackermann, M., Engels, M., 2006. Pro and contra IBR-eradication. Vet. Microbiol. https://doi.org/10.1016/j.vetmic.2005.11.043

Barnard, B.J.H., 1997. Antibodies against some viruses of domestic animals in southern African wild animals. Onderstepoort J. Vet. Res.

Biswas, S., Bandyopadhyay, S., Dimri, U., H. Patra, P., 2013. Bovine herpesvirus-1 (BHV-1) - a re-emerging concern in livestock: A revisit to its biology, epidemiology, diagnosis, and prophylaxis. Vet. Q. https://doi.org/10.1080/01652176.2013.799301

Böttcher, J., Boje, J., Janowetz, B., Alex, M., König, P., Hagg, M., Götz, F., Renner, K., Otterbein, C., Mages, J., Meier, N., Wittkowski, G., 2012. Epidemiologically non-feasible singleton reactors at the final stage of BoHV1 eradication: Serological evidence of BoHV2 cross-reactivity. Vet. Microbiol. https://doi.org/10.1016/j.vetmic.2012.04.017

Celeste, J.L. de L., Caldeira, R.L., Pires, S. da F., Silveira, K.D., Soares, R.P., de Andrade, H.M., 2019. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of Leishmania amazonensis in skin samples. Exp. Parasitol. https://doi.org/10.1016/j.exppara.2019.05.006

d'Offay, J.M., Fulton, R.W., Fishbein, M., Eberle, R., Dubovi, E.J., 2019. Isolation of a naturally occurring vaccine/wild-type recombinant bovine herpesvirus type 1 (BoHV-1) from an aborted bovine fetus. Vaccine. https://doi.org/10.1016/j.vaccine.2019.06.059

Ek-Kommonen, C., Pelkonen, S., Nettleton, P.F., 1986. Isolation of a herpesvirus serologically related to bovine herpesvirus 1 from a reindeer (Rangifer tarandus). Acta Vet. Scand.

El-Kholy, A.A., Abdelrahman, K., Soliman, H., 2014. Rapid detection of BoHV-1 genomic DNA by loop-mediated isothermal amplification assay. J. Virol. Methods. https://doi.org/10.1016/j.jviromet.2014.04.011

Fan, Q., Xie, Zhixun, Xie, Zhiqin, Xie, L., Huang, J., Zhang, Y., Zeng, T., Zhang, M., Wan, S., Luo, S., Liu, J., Deng, X., 2018. Development of duplex fluorescence-based loop-mediated isothermal amplification assay for detection of Mycoplasma bovis and bovine herpes virus 1. J. Virol. Methods. https://doi.org/10.1016/j.jviromet.2018.08.014

French, E.L., 1962. A specific virus encephalitis in calves: Isolation and characterization of the causal agent. Aust. Vet. J. https://doi.org/10.1111/j.1751-0813.1962.tb16043.x

Gabev, E., Fraefel, C., Ackermann, M., Tobler, K., 2009. Cloning of Bovine herpesvirus type 1 and type 5 as infectious bacterial artifical chromosomes. BMC Res. Notes. https://doi.org/10.1186/1756-0500-2-209

Gatesy, J., Amato, G., Vrba, E., Schaller, G., Desalle, R., 1997. A Cladistic Analysis of Mitochondrial Ribosomal DNA from the Bovidae. Mol. Phylogenet. Evol. https://doi.org/10.1006/mpev.1997.0402

Geiger, R., Munz, E., Hübschle, O.J.B., Reimann, M., 1990. Seroepizootiologische Untersuchungen über die Verbreitung von Bovinem Herpesvirus Typ 1 und 2 (BHV 1, BHV 2) in Namibia. J. Vet. Med. Ser. B. https://doi.org/10.1111/j.1439-0450.1990.tb01047.x

Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K.I., 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. Biotechniques. https://doi.org/10.2144/000113072

Inglis, D.M., Bowie, J.M., Allan, M.J., Nettleton, P.F., 1983. Ocular disease in red deer calves associated with a herpesvirus infection. Vet. Rec. https://doi.org/10.1136/vr.113.8.182

Kendrick, J.W., Gillespie, J.H., Mcentee, K., 1958. Infectious pustular vulvovaginitis of cattle. Cornell Vet.

Kool, E.T., 2001. Hydrogen bonding, base stacking, and steric effects in DNA replication. Annu. Rev. Biophys. Biomol. Struct. https://doi.org/10.1146/annurev.biophys.30.1.1

Maidana, S.S., Craig, P.O., Craig, M.I., Ludwig, L., Mauroy, A., Thiry, E., Romera, S.A., 2017. Evidence of natural interspecific recombinant viruses between bovine alphaherpesviruses 1 and 5. Virus Res. https://doi.org/10.1016/j.virusres.2017.09.018

Martin, J.R., Harvey, D., Montpetit, C., 1987. La mammillite herpétique bovine au Québec. Can. Vet. J. = La Rev. Vet. Can.

Mayfield, J.E., Good, P.J., VanOort, H.J., Campbell, A.R., Reed, D.E., 1983. Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (Cooper strain). J. Virol. https://doi.org/10.1128/jvi.47.1.259-264.1983

Metzler, A.E., Schudel, A.A., Engels, M., 1986. Bovine herpesvirus 1: Molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. Arch. Virol. https://doi.org/10.1007/BF01315300

Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Thiry, E., 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. Vet. Res. https://doi.org/10.1051/vetres:2006059

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes.

https://doi.org/10.1006/mcpr.2002.0415

Nandi, S., Kumar, M., Manohar, M., Chauhan, R.S., 2009. Bovine herpes virus infections in cattle. Anim. Health Res. Rev. https://doi.org/10.1017/S1466252309990028

Nettleton, P., Russell, G., 2017. Update on infectious bovine rhinotracheitis. In Pract. https://doi.org/10.1136/inp.j2226

Notomi, T., 2000. Loop-medicated isothermal amplification of DNA. Nucleic Acids Res.

Notomi, T., Mori, Y., Tomita, N., Kanda, H., 2015. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. J. Microbiol. https://doi.org/10.1007/s12275-015-4656-9

OIE, 2013. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organ. Anim. Heal. https://doi.org/10.1007/s13398-014-0173-7.2

Oscorbin, I.P., Belousova, E.A., Zakabunin, A.I., Boyarskikh, U.A., Filipenko, M.L., 2016. Comparison of fluorescent intercalating dyes for quantitative loop-mediated isothermal amplification (qLAMP). Biotechniques. https://doi.org/10.2144/000114432

Pawar, S.S., Meshram, C.D., Singh, N.K., Sonwane, A.A., Saini, M., Rautmare, S.S., Muglikar, D.M., Mishra, B.P., Gupta, P.K., 2014. Rapid detection of bovine herpesvirus 1 in bovine semen by loop-mediated isothermal amplification (LAMP) assay. Arch. Virol. https://doi.org/10.1007/s00705-013-1869-2

Schwyzer, M., Ackermann, M., 1996. Molecular virology of ruminant herpesviruses. Vet. Microbiol. https://doi.org/10.1016/S0378-1135(96)01231-X

Socha, W., Rola, J., Urban-Chmiel, R., Zmudziński, J.F., 2017. Application of Loop-Mediated Isothermal Amplification (LAMP) assays for the detection of bovine herpesvirus 1. Pol. J. Vet. Sci. https://doi.org/10.1515/pjvs-2017-0078

St George, T., Philpott, M., 1972. Isolation of Infectious Bovine Rhinotracheitis virus from the prepuce of water buffalo bulls in Australia. Aust. Vet. J. https://doi.org/10.1111/j.1751-0813.1972.tb02244.x

Stahel, A.B.J., Baggenstos, R., Engels, M., Friess, M., Ackermann, M., 2013. Two different Macaviruses, ovine herpesvirus-2 and caprine herpesvirus-2, behave differently in water buffaloes than in cattle or in their respective reservoir species. PLoS One. https://doi.org/10.1371/journal.pone.0083695

Tanner, N.A., Evans, T.C., 2013. Loop-mediated isothermal amplification for detection of nucleic acids. Curr. Protoc. Mol. Biol. https://doi.org/10.1002/0471142727.mb1514s105

Tanner, N.A., Zhang, Y., Evans, T.C., 2015. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. Biotechniques. https://doi.org/10.2144/000114253

Thiry, J., Keuser, V., Muylkens, B., Meurens, F., Gogev, S., Vanderplasschen, A., Thiry, E., 2006. Ruminant alphaherpesviruses related to bovine herpesvirus 1. Vet. Res. https://doi.org/10.1051/vetres:2005052

Trevethan, R., 2017. Sensitivity, Specificity, and Predictive Values: Foundations, Pliabilities, and Pitfalls in Research and Practice. Front. Public Heal. https://doi.org/10.3389/fpubh.2017.00307

Vingataramin, L., Frost, E.H., 2015. A single protocol for extraction of gDNA from bacteria and yeast. Biotechniques. https://doi.org/10.2144/000114263

Appendix

Supplementary Table A

Detailed information on the clinical samples tested.

Sample number	Date arrived	Species	Age	sample type	extraction method and date	BoHV-1	BPI3	BRSV	BCoV
	IBR neg								
1	28. Nov 17	Bovine		Tracheal swab	Arrow Viral NA 29Nov17		33,78	19,83	
2	29. Nov 17	Bovine	5 months	Swab	Arrow Viral NA 06Dec17			42,96	23,96
3	29. Nov 17	Bovine	5 months	Swab	Arrow Viral NA 06Dec17				
4	06. Mär 18	Bovine		Swab	Arrow Viral NA 07Mar18				
5	06. Mär 18	Bovine		Swab	Arrow Viral NA 07Mar18				
6	04. Dez 18	Bovine	9 months	swab	Arrow Viral NA 05Dec18			23,68	
7	04. Jan 19	Bovine	4 months	Tracheal swab	Arrow Viral NA 09Jan19			17,02	
8	15. Apr 19	Bovine	6 months	swab	GXT NA 18Apr19 1/2				
9	15. Apr 19	Bovine	6 months	swab	GXT NA 18Apr19 1/2				
10	unknown	Bovine	unknown	swab	GTX NA 29jul19				
11	unknown	Bovine	unknown	swab	GTX NA 29jul19				
12	unknown	Bovine	unknown	swab	GTX NA 29jul19				
13	unknown	Bovine	unknown	swab	GTX NA 29jul19				
14	unknown	Bovine	unknown	pharyngeal sw	GTX NA 29jul19				
	IBR pos								
15	28. Nov 17	Bovine	unknown	Tracheal swab	Arrow Viral NA 29Nov17	30,49			
16	28. Nov 17	Bovine		Tracheal swab	Arrow Viral NA 29Nov17	40,33		43,26	38,11
17	29. Nov 17	Bovine	5 months	Swab	Arrow Viral NA 06Dec17	40,56			28,96
18	18. Okt 17	Bovine		Swab	Arrow Viral NA 01Nov17	37,04			
19	18. Okt 17	Bovine		Swab	Arrow Viral NA 01Nov17	32,08		43,47	
20	08. Nov 18	Bovine	14 months	Tracheal swab	Arrow Viral NA 14Nov18	14,81	29,58	33,08	
21	13. Nov 18	Bovine		Tracheal swab	Arrow Viral NA 14Nov18	16,36		26,77	
22	13. Nov 18	Bovine		Tracheal swab	Arrow Viral NA 14Nov18	19,76		21,12	
23	11. Jan 19	Bovine	6 months	swab	Arrow Viral NA 16Jan19	18,11			
24	11. Jan 19	Bovine	9 months	swab	Arrow Viral NA 16Jan19	19,4			
25	11. Jan 19	Bovine	8 months	swab	Arrow Viral NA 16Jan19	16,42			
26	11. Jan 19	Bovine	7 months	swab	Arrow Viral NA 16Jan19	18,1			
27	14. Feb 19	Bovine		Nasal swab	Arrow Viral NA 19Feb19	31,94			

Sample number	Mycoplasma	Mycoplasma bovis	Histophilus so	Past multocida	Mannheimia hae	Control	Spike 1	IBR status	IBR status qPCR Zurich (Ct)
1	18,44	21,84	18,95	26,24		20,36	22,96	IBR neg	
2	37,31		15,37	31,17	23,07	16,2	22,7	IBR neg	
3	38.91		21.94	30.64	29.13	17,84	23. Jan	IBR neg	
4						18,98	22,9	IBR neg	
5						18,39	22,88	IBR neg	
6	30,12					19,17	22,34	IBR neg	
7	32,95	14,59		22,39	26,09	16,46	22,07	IBR neg	
8	25,23	26,09	20,48	25,04	19,41	16,15	21,7	IBR neg	
9	32,28	28,98	19,23	19,92	30,79	16,29	22,47	IBR neg	
10			31,72	23,48	26,66	17,73	22,72	IBR neg	
11	32,52		29	18,02	24,99	16,23	22,15	IBR neg	
12			19,65	17,73		19,03	22,43	IBR neg	
13		31,66				15,9	22,78	IBR neg	
14	30,04		29,36	25,73		16,95	22,63	IBR neg	
15	13,13	11,67	17,4	23,05	21,24	19,62	22,89	IBR pos	17,033
16	14,48	16,28	19,4	20,99	32,11	17,35	22,64	IBR pos	
17	20,83		17,97	25,31	21,37	18,27	23,17	IBR pos	
18	39,29			42,95	27,31	20,93	22,78	IBR pos	30,943
19			42,96			19,57	22,73	IBR pos	29,194
20	10,69	11,52		28,42	28,22	19,01	22,99	IBR pos	26,486
21	12,91	12,84	20,27	25,56	20,02	21,12	23,36	IBR pos	21,896
22	11,58	12,59	21,31	27,53	20,55	19,83	22,35	IBR pos	23,766
23	26,06	32,59	28,04	24,18	22,58	18,99	22,23	IBR pos	18,357
24	32,19	33,01	30,02	31,05		18,31	22,09	IBR pos	24,615
25	33,04	19,84	22,32			19,37	22,16	IBR pos	21,03
26		30,53	28,89			21,52	22,44	IBR pos	22,389
27						15,83	21,88	IBR pos	

Supplementary Table B

List of evaluated primer sets. The genome position refers to the position of the LAMP amplicon and is relative to the reference sequence for BoHV-1 (GenBank accession number NC001847.1).

Target gene Genome position	**Primer**	**Sequence**	**GC content amplicon**
gB	Fan et al., 2018		65.4%
gC	Pawar et al., 2014		66.9%
gD	Socha et al., 2017		65.4%
gE	Socha et al., 2017		65.1%
Pol [U_L 30]	F3	5'-CAGCTTGAGCTTCTCCAC-3'	60.2%
47'305 – 47'555	B3	5'-CGTTCCTCAAGCAGTACTC-3'	
	FIP (F1c+F2)	5'-CCTCTTCCGCGTCTTCGACACCACGCCGTTAATCTTC-3'	
	BIP (B1c+B2)	5'-GCCGTGAGCTTCTCCGAGAGTCACCGGGTACAACATC-3'	
	LF	5'-TTCAGAAGCAGAGCAAGGT-3'	
	LB	5'-GTACGCCCAGTCGAAGTT-3'	
Hel [U_L 5]	F3	5'-TCACCTGCCTTATCACCA-3'	61.8%
94'627 – 95'071	B3	5'-TGCGAGTAGTTGGTAATGC-3'	
	FIP (F1c+F2)	5'-TTGTTAATGAAGGCGTCTGGCATGCCAGGAGTACGAGTTC-3'	
	BIP (B1c+B2)	5'-CTACATGAGCCGCCTCCACGAAGGTGCGGATGTTGAC-3'	
	LF	5'-GAGGTGCTCTTCCGTCAC-3'	
	LB	5'-TTGTGGTCTTTACGCTACCC-3'	
V67 [U_S 1.67]	F3	5'-GTACTGGCTCATGTTTCCC-3'	61.8%
114'483 – 114'731	B3	5'-CTGCTGGTGAAAGTTCCC-3'	
	FIP (F1c+F2)	5'-TCGTTAAGCTTCCGCACAACTGTTGAGGTAGAAGCGGTC-3'	
	BIP (B1c+B2)	5'-TGTTCCGTCGTAAAGCTGACGGTTCACTTTGAATGTGTTCCC-3'	
	LF	5'-AGACGAGTGCTACGAGGA-3'	
	LB	5'-AAAGAACTGCAGCGGTCG-3'	

F3 = outer forward primer; B3 = outer backward primer; FIP = forward inner primer; BIP = backward inner primer; LF = forward loop primer; LB = backward loop primer; Pol = Polymerase, catalytic subunit; Hel = Helicase, V67 = tegument protein V67; U_L = unique long sequence; U_S = unique short sequence.

Supplementary Figure A

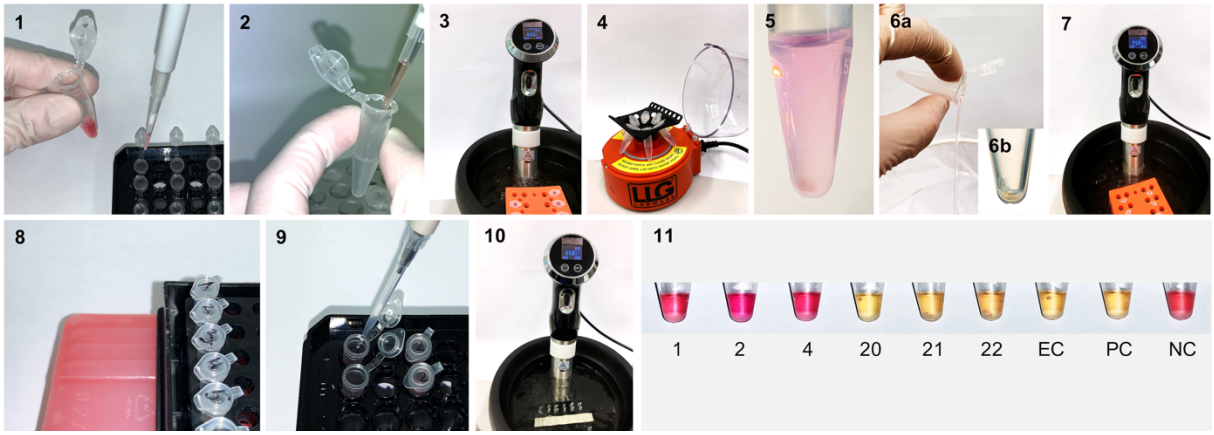

Illustration of a possible set up using limited equipment. ***1*** *Prepare PCR tubes by filling in 23 µl reaction mix containing 2x WarmStart® Colorimetric master mix, 10x primer mix and DEPC treated water.* ***2*** *Add 100 µl of sample to 455 µl of DNA extraction solution in a 1.5 ml Eppendorf tube.* ***3*** *Incubate at 80°C for 10 min by placing tubes in the Styrofoam tube holder, which floats on the surface of the water bath.* ***4*** *Centrifuge at 2000 x g for 3 min.* ***5*** *A pellet containing the DNA is visible in most of the cases. Sometimes, however, the pellet is not clearly visible.* ***6*** *Discard extraction solution (**6a**) and assure to remove all remaining liquid (**6b**), because the alkalinity of the remaining buffer inhibits color change during LAMP. Then, re-suspend the pellet in 60 µl of DEPC treated water.* ***7*** *Dilute the re-suspended pellet 1:5 in DEPC treated water (4 µl of sample in 16 µl of DEPC treated water) in a PCR tube, place the tubes in the Styrofoam tube holder, and incubate at 95°C for 5 min for denaturation.* ***8*** *Place denatured samples on a cold pack.* ***9*** *Add 2 µl of denatured sample to the already prepared reaction mix in the reaction tubes (see **1**).* ***10*** *Incubate samples at 65°C by weighing down the tube holder and evaluate color of the mix on white background after 30 min.* ***11*** *yellow = positive result; purple = negative result. Samples 1 – 3 where known to be negative for BoHV-1, and also gave a negative result in this simple setting. Samples 4 – 6 where known to be positive, and also gave a positive result in this simple setting. Sample 7 is a positive extraction control, sample 8 a positive amplification control and sample 9 the negative control consisting of DEPC treated water. Samples were tested in duplicates, but as all the duplicates showed identical results, only one tube is shown.*

ACKNOWLEDGEMENTS

This work was funded by the Federal Food Safety and Veterinary Office (BLV, project number 1.18.03).

I would like to thank everyone who contributed to this dissertation, especially PD Dr. med. vet. Claudia Bachofen for her great supervision, support and patience during all this time.

I also thank Prof. Dr. Cornel Fraefel for giving me the opportunity to work at the institute and for his scientific inputs.

Further, I would also like to thank Prof. Dr. Mathias Ackermann and Kurt Tobler for their scientific support and advice.

Many thanks to the Virus Surveillance Unit from the Moredun Research Institute in Scotland for providing us with clinical samples from BoHV-1 infected cattle – especially Madeleine Maley for selecting and shipping the samples and providing us with detailed information on the specimen.

A big thank you to all the members of the institute, especially my group members Isabelle H., Charlotte, Karin, Tamara, Jakub and Isabelle V., and Julia, Marco and Martina from diagnostics and Anita for their help, the motivating atmosphere, the laughs and the numerous lessons on equine behaviour.

Last but not least, I thank my family and my friends Kathrin, Regula, Tina, Carmen and Eliane for always supporting me and believing in me.

CURRICULUM VITAE

Vorname Name	Deborah Peltzer
Geburtsdatum	12.05.1991
Geburtsort	Rheinfelden, AG, Schweiz
Nationalität	Schweiz, Deutschland
Heimatort	Basel-Stadt, BS
August 1998 – Juni 2003	Primarschule, Stein (AG), Schweiz
August 2003 – Juni 2007	Bezirksschule, Rheinfelden (AG), Schweiz
August 2007 – Dezember 2011	Gymnasium, Muttenz (BL), Schweiz
12. Dezember 2011	Matura, Gymnasium Muttenz (BL), Schweiz
September 2013 – Dezember 2018	Studium der Veterinärmedizin, Universität Zürich, Schweiz
28. Dezember 2018	Staatsexamen med. vet.**,** Universität Zürich, Schweiz
Februar 2019 – August 2020	Anfertigung der Dissertation unter Leitung von PD Dr. med. vet. Claudia Bachofen am Virologischen Institut der Vetsuisse-Fakultät Universität Zürich Direktor Prof. Dr. sc. nat. ETH Cornel Fraefel

Ort, Datum	Signatur
Zürich, 12.11.2020